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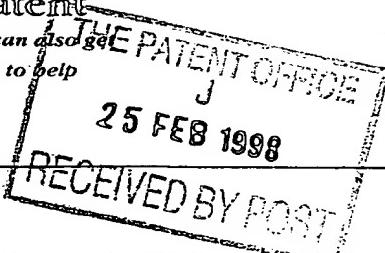
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BIOVATION LIMITED
AURIS BUSINESS CENTRE
23 ST MACHAR DRIVE
ABERDEEN AB24 3RY
0658 4809003.

4. Title of the invention

RIBOSOME DISPLAY

5. Name of your agent (if you have one)

Kilburn & Strode
20 Red Lion Street
~~As in (3) above~~ London
WC1R 4PJ
see 51/77
filed 22/9/48

Patents ADP number (if you know it)

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RIBOSOME DISPLAY

The present invention relates to methods and compositions for the generation and screening of combinatorial libraries of displayed polypeptides encoded by natural or artificial DNA sequences which are expressed on polysomes following *in vitro* transcription and translation. In particular, the invention relates to the efficient expression of full-length polypeptides on polysomes attached to the encoding mRNA sequence and the subsequent determination of that sequence.

The ribosome represents a collection of proteins whose co-ordinated activities accomplish the act of translation. The basic form of the ribosome is conserved, though there are appreciable differences in the size and proportions of RNA and proteins in the ribosomes of prokaryotes and eukaryotes, and in organelles. All ribosomes consist of two main subunits, in bacteria a 50S and 30S, in eukaryotes a 60s and 40S subunit. Protein synthesis starts when the ribosome attaches to mRNA either at the 5' cap in eukaryotes or adjacent to the translational initiation codon in prokaryotes (usually AUG) and continues by the successive loading, directed by the mRNA sequence, of amino acids onto a peptidyl-tRNA molecule carrying the nascent polypeptide chain. Translation terminates usually at a stop codon where the ribosome disassociates from the mRNA. In the absence of a stop codon, the ribosome is thought to progress to the end of the mRNA molecule before disassociation occurs with the assistance of release factors. Translation termination therefore disconnects the protein from the mRNA molecule encoding it. If the ribosome is arrested during protein synthesis, then the protein and mRNA will remain connected whilst complexed with the ribosome.

Methods of selecting proteins as the carrier of a particular phenotype and subsequently determining the corresponding genotype have relied mainly on living cells to provide a link between genes and proteins commonly using bacteriophage, viruses and bacterial cells displaying the desired protein. For a diverse collection of proteins represented by a DNA library, the use of live cells has several disadvantages. For example, the protein diversity can be reduced by the requirement for transformation or infection of bacterial or eukaryotic cells which is limited by the low efficiency of DNA or infectious particle uptake. Furthermore, the biological production of diverse proteins is subject to the particular environment of the living cell which can select against certain proteins and can lead to the variations in protein folding which can also select against certain proteins. Finally, if diversification of individual proteins during successive selection rounds is required, genetic mutation techniques are difficult to apply to *in vivo* systems due to the need to switch between DNA and live cells for the diversification and screening.

In vitro methods for the selection of a polypeptide potentially offer advantages over *in vivo* methods by eliminating the need for uptake of genes into cells and by controlling the environment for mRNA and protein production. *In vitro* transcription and translation reactions have been used for many years as a means of generating polypeptides directly from DNA and it has been shown that specific mRNAs can be enriched by the immunoprecipitation of polysomes (e.g. Payvar, F. and Schimke, R. T., Eur. J. Biochem., vol 101 (1979) p1844-1848) using antibodies to select the specific polypeptides. This so-called "ribosome display" technique has more recently been

adapted for the selection of peptides (Mattheakis, L. *et al*, PNAS 91: 9022, 1994 and PCT95/11922) and proteins (Kawasaki, G., PCT91/05058 and Hanes & Pluckthun, PNAS, vol 94 [10]:4937, 1997).

The method described by Mattheakis, L. *et al* (PNAS 91: 9022, 1994) uses ribosome display systems for identification of ligands from peptide libraries. The method uses chloramphenicol for ribosome translation arrest which induces stalling in ribosome translation by binding the 50S subunit of the prokaryotic ribosome complex. A disadvantage of this method is that it will cause translation arrest regardless of the length of the nascent peptide and thus most polypeptide molecules will be incomplete. For efficient screening of polypeptides from a DNA library following transcription/translation, it is clearly desirable to maximise the yield of full-length polypeptides associated with mRNA.

The methods described by Mattheakis and Dower in PCT95/11922 include additional measures to stall ribosomes principally through the use of "Tethered Nascent Peptides" which are peptide portions of a fusion polypeptide molecule, adjacent to the polypeptide portion for screening, which interact or bind with the encoding polynucleotide in order to maximise the yield of polypeptides associated with mRNA. As with Mattheakis, L. *et al* (PNAS, ibid), this method would be expected to cause premature translational arrest and would also be expected to distort the proper folding of some proteins for screening. Thus, a range of sizes of polypeptide molecules would be expected by the methods described by Mattheakis and Dower which would reduce the probability of isolating specific polypeptides encoded by the DNA library.

In the method of Hanes & Pluckthun (ibid), there is provided a method to optimise the yield of correctly folded protein and its encoding mRNA while both are still attached to the ribosome. The method includes stalling the translation process by increasing magnesium acetate concentration and correct folding of proteins by the manipulation of the physical reaction conditions. However, the only measure to increase the yield of full-length protein is to eliminate stop codons from the mRNA by manipulating the corresponding genes and adding a 3' spacer region in order to tether the folded protein on the ribosome. Whilst this method should increase the proportion of full-length polypeptides when compared to earlier methods, the method provides no measures to prevent run-off translation from the end of the mRNA and the stalling strategies provided suffer from the same limitations as with Mattheakis and Dower (ibid) whereby premature translational arrest would be expected.

In addition to the limitations described for each of the methods above, none of the methods provide a means to block new translational starts late in the translation reaction prior to testing of translated polypeptides. Thus, a range of sizes of polypeptide molecules would be expected by these methods which would reduce the probability of isolating specific polypeptides encoded by the DNA library. There is therefore a need for new methods for ribosome display which maximise the yield of full-length polypeptides whilst minimising the presence of nascent incomplete polypeptide chains in order to efficiently screen a DNA library for specific polypeptides.

The present invention provides a new *in vitro* method of ribosome display which maximises the expression of full-length polypeptides from a ribosome by selective arrest of mRNA translation at the 3' end of the mRNA such that the ribosome complex it is still linked to the full-length polypeptide. The invention is based on the discovery that proteins bound to specific sites in mRNA molecules will block the further translation of the mRNA allowing the stalling of a mRNA-ribosome complex with associated polypeptide. The invention also includes optional measures to prevent new translational starts just prior to polypeptide screening.

The method of the present invention involves firstly the creation of a library of DNA molecules, commonly within a plasmid vector, such DNA molecules encoding various polypeptides whereby the DNA encoding these polypeptides is transcribed into mRNA molecules. Commonly, the cloning vector for the DNA molecules includes an upstream promotor such as a promotor for T7 RNA polymerase. Thus, the pooled library of DNA molecules is transcribed, for example by the addition of T7 RNA polymerase and ribonucleotides, to produce a library of mRNA molecules. Thereafter, the library of RNA molecules is translated using a ribosome preparation such as an E.coli S-30 fraction (Chen H Z and Zubay G, Methods in Enzymology, vol 101 (1983) p674-690). Thereafter, the ribosome complexes, comprising linked polypeptide, ribosome and mRNA, are typically screened for binding to a ligand immobilised onto a solid phase and mRNA is released from the resultant immobilised complexes for reverse transcription and amplification by PCR in order to enrich for DNA molecules encoding polypeptides which bind to the target ligand. These DNA molecules can either be used directly for transcription or can be cloned in order to determine the sequences of DNA encoding polypeptides which bind to the target ligand.

For the purpose of this invention, the mRNA encoded by the DNA molecules will be considered to include 3 segments comprising, from 5' to 3', a variable segment, a spacer segment and a termination segment with an optional anti-initiation segment 5' to the variable segment. The polypeptide shall mean the protein or peptide sequence translated from the variable segment of the mRNA to protein via a ribosome complex. The variable segment shall mean mRNA sequences encoding the full-length polypeptide. The spacer segment shall mean mRNA sequences encoding a protein segments contiguous with the variable segments which allow the completed proteins to completely emerge from the ribosome and adopt optimal three dimensional structures whilst still attached to the encoding mRNA through the ribosome complex. The terminating segment shall mean mRNA sequences to which the binding moiety attaches either directly or indirectly or, alternatively, mRNA sequences encoding a polypeptide binding moiety, contiguous with or upstream from the spacer segment to which specific proteins attach in order to block translation. The binding moiety shall mean any molecule that can bind either to the mRNA on the ribosomal complex or any molecule that can bind to the translated polypeptide on the ribosomal complex resulting in arrest of translation. The binding moiety will usually bind directly to mRNA in a sequence specific manner or may be bound indirectly to the mRNA, for example after annealing of a synthetic DNA or RNA molecule to the mRNA and addition of the binding moiety via a ligand on these molecules. The optional anti-initiation segment will be adjacent to the translational initiation codon and will provide sequences for attachment usually of a different binding moiety. The anti-initiation segment will prevent new translational initiations just prior to screening of the

translated polypeptides. The target ligand will be the molecule against which the library is screened for binding of specific proteins and subsequent recovery of associated mRNA.

The variable segment of the mRNA molecules comprises either full-length known polypeptide types such as single-chain antibodies (comprising immunoglobulin variable regions derived from heavy and light chains and linked together to give a functional binding domain), or random or semi-random sequences. Chimaeric sequences created by random/semi-random association of known polypeptide types or regions may also be used. For known polypeptide types such as single-chain antibodies, specific segments of the genes may be randomised such as the CDRs in single-chain antibodies. Large collections of DNA molecules encoding known, random or semi-random sequences will be firstly produced and then cloned into the transcription vector. This transcription vector will provide other segments for inclusion in the subsequent mRNA molecules as detailed below. The vector will also usually provide a translation initiation codon and ribosome binding site for the mRNA. For longer polypeptide molecules encoded by the DNA, it will be necessary to reduce or eliminate the presence of stop codons in the mRNA which would terminate translation. It is a requirement of the invention herein that such stop codons, including stop codons natural to specific proteins, are eliminated in order to yield full-length polypeptides. For DNA encoding known proteins such as single-chain antibodies, this will simply require elimination of the usual stop codon or, alternatively, the use of nonsense suppressing tRNAs in the translation reaction in order to insert specific amino acids at the position of the stop codons. For random or semi-random DNA produced by chemical synthesis, the frequency of stop codons occurring in any particular sequence can be reduced by manipulation of DNA base composition in the synthesis reaction and nonsense suppressing tRNAs could also be used in the translation reaction.

The spacer segment of the mRNA molecules provides a polypeptide region downstream of the variable segment which spans the channel of the ribosome in order to permit the full-length polypeptide to fully emerge from the ribosome to allow for proper protein folding and unhindered access to the target ligand. Usually this segment will encode a region of over 50 amino acids and will encode a region which is unlikely to interfere with folding of the full-length polypeptide such as a complete domain from another protein or a glycine/alanine-rich linker such as (Gly₄Ser)₁₀. For some translated proteins, the spacer segment may comprise a contiguous portion from the protein itself where this portion is not required for correct folding of the variable segment or for binding to the target ligand.

The terminating segment provides a downstream mRNA sequence for direct or indirect attachment of a binding moiety which is designed to prevent translational termination following translation of the complete variable segment. One example of a binding moiety which binds to a terminating segment in mRNA is the Iron Regulatory Protein (IRP). The terminating segment would provide a stem-loop structure which is stabilised by the addition of the IRP in conditions of low iron concentration, thus resulting in a steric block to the ribosome translation. Following this selection of the nascent peptide can occur. The use of IRP to arrest the ribosome introduces a reversible block such that addition of iron may allow translation to resume and thus terminate. This would facilitate the release of the mRNA after selection of the

polypeptide for subsequent transcription, sequencing or cDNA cloning. Further examples of mRNA binding moieties binding to sequences in the terminating segment include the HIV protein tat, which binds to a RNA stem-loop termed TAR (Dingwall et al., PNAS, vol 86 (1989) p6925-6929), La antigen which binds to a RNP motif (Chan E K L and Tan E M, Mol. Cell. Biol. vol 7 (1987) p2588-2591) and other proteins from RNA viruses which bind to specific RNA sequences either in single- or double-stranded RNA, the latter which can be created in mRNA via hairpin loops. The terminating segment alternatively could encode a site for attachment of a binding moiety to the polypeptide in order to prevent translational termination. Optionally, the terminating segment will include one or more further regions of mRNA which increase the stability of mRNA against exonucleases such as the lpp (E.coli lipoprotein) and phage T3 terminators.

The optional anti-initiation segment adjacent is designed to prevent translational initiations late in the translation reaction which might not result in full-length polypeptides and might therefore provide polypeptides with incomplete folding which might provide non-specific binding to target ligands. The anti-initiation segment might encode, for example, a secretory leader sequence in the translated polypeptide to which can be bound the signal recognition protein (SRP). Once SRP has bound to the polypeptide, it causes the arrest of further translation by cross-linking of the polypeptide and the mRNA. Translational arrest may also be achieved by using a combination of the SRP54 and SRP9/14 subunits of SRP (Siegel & Walter, Cell Biol., vol 100 (1985) 1913-1921) which bind to the nascent peptide and mRNA respectively. Another example of anti-initiation sequences is provided by certain eukaryotic transcription leaders such as that of the human cytomegalovirus gp48 gene which contains an upstream 22 codon which represses translation of the downstream cistron (Cao J and Geballe A P, Mol. Cell. Biol. vol 16 (1996) p7109-7114). Such leaders are thought to encode peptides which block ribosome progression to the downstream cistron. Further examples of anti-initiation sequences are sequences recognised by binding moieties such as for the terminating segment as above, these including sequences bound by IRP, tat, La antigen and other viral proteins.

The invention therefore provides compositions of DNA libraries encoding mRNA molecules with variable, spacer and terminating segments with an optional anti-initiation segment. Preferably, the invention provides a DNA vector encoding the spacer and terminating segments with the optional anti-initiation segment. Commonly, the DNA vector will also provide the translation initiation codon and, for translations using prokaryotic ribosomes, a ribosome binding site including the Shine-Dalgarno sequence. For eukaryotic translation systems, the Kozak translation initiation sequence with consensus GCCGCCACCATGG may also be included and, upstream from the translation initiation site, it may be desirable to include other known sequences to enhance translation such as enhancers or activator sequences including untranslated leader sequences from certain viruses such as tobacco mosaic virus. Commonly, the DNA vector will also provide a strong transcriptional promotor which will be used in conjunction with a RNA polymerase to produce a library of mRNA molecules corresponding to the DNA library. Such promotors will include those for T7 RNA polymerase, T3 RNA polymerase and SP6 RNA polymerase and, additionally, a promotor for the RNA dependant polymerase, Qb replicase, may also be encoded by the DNA. The DNA vector will also provide a strong transcriptional

terminator, for example the terminator of *E. coli* lipoprotein or the early terminator of phage T3. In the method of the invention, DNA fragments containing the variable segments are cloned into the DNA vector whereby the DNA fragments have a minimum of or no stop codons. For libraries encoding known polypeptide types such as single-chain antibodies, the DNA fragments will be cloned unidirectionally using appropriate restriction sites. It will be apparent to those skilled in the art that a variety of replicable DNA vectors could be used in the method of the present invention including plasmid, bacteriophage, phagemid and viral vectors. It will also be clear that DNA amplification by methods such as PCR could be used as an alternative to replication of DNA in living cells. It will also be clear that the variable segments to create the DNA library could be provided from a number of sources including a vector library of DNA fragments, synthetic DNA or amplified DNA. In addition, the variable segments could be provided as a result of mutagenesis reactions using a fixed template, for example using error-prone PCR. It will also be clear that the variable segments could be composed of DNA directly from the genome of a living organism or from cDNA copies of mRNAs of that organism. For example, where the DNA library comprises single-chain antibody fragments, these fragments could be derived from mRNA encoding immunoglobulin variable regions and expressed by B cells within an organism. Alternatively, the fragments could be derived from genomic variable regions. In such manner, the invention will provide for the creation of single-chain antibody libraries from specific organisms such as man.

The invention also provides compositions of libraries of mRNA molecules with variable, spacer and terminating segments with an optional anti-initiation segment. Preferably, the invention provides a library of mRNA molecules encoding the spacer and terminating segments with the optional anti-initiation sequence. The mRNA molecules will each include a translation initiation codon and, for translations using prokaryotic ribosomes, a ribosome binding site including the Shine-Dalgarno sequence. For translation using eukaryotic ribosomes, the mRNA may be synthesised with a 5' capping nucleotide or this may be introduced enzymatically into the pre-synthesised mRNA. Upstream from the translation initiation site, the consensus Kozak translation initiation sequence may also be included and other known sequences to enhance translation such as enhancers or activator sequences including untranslated leader sequences from certain viruses. Prior to or during the process of translation, one or more binding moieties will become associated with the terminating segment of the mRNA molecules thus stalling translation. Also, during the process of translation, one or more binding moieties may become associated with the optional anti-initiation segment within or encoded by the mRNA molecules thus preventing new translational initiations.

The invention also provides compositions of libraries of translated polypeptide molecules with variable and spacer segments with an optional anti-initiation leader sequence. These protein molecules will provide full-length variable segments as part of a longer polypeptide chain which is stalled within the ribosomes through the interaction of binding moieties with the mRNA. At the 5' end of the polypeptide chain, there may also be a site for association of a binding moiety, such as SRP, which interacts with the ribosome thus preventing additional translation on the mRNA molecule.

Within the method of the present invention, it will be apparent to those skilled in the

art that various measures could be used to optimise the stability of mRNA molecules especially from degradation by RNases. For example, various inhibitors of RNases such as Rnasin and vanadyl ribonucleoside complexes could be used in the transcription reactions. Alternatively or additionally, various structures could be included in the mRNA molecules including 3' stem-loops such as those commonly provided by transcriptional terminators. It will also be apparent to those skilled in the art that transcription and translation reactions might either be performed separately or, especially with prokaryotic systems, combined into a coupled *in vitro* transcription/translation reaction. Preferably, the transcription and translation reactions will be performed separately in order to optimise the production of mRNA and polypeptides which will require different optimal reagents.

Within the method of the present invention, it will be apparent to those skilled in the art that various measures could be used to optimise the folding and stability of protein molecules especially from degradation by proteases. For correct protein folding, optimal oxidative protein folding conditions would be used in translation reactions with particular measures to optimise disulphide bond formation through, for example, use of molecular chaperones such as protein disulphide isomerase. For stability, the peptide tagging system of E.coli could be disabled by inhibition of *ssrA* RNA using methods such as that of Hanes and Pluckthun (*ibid*). Once the final polypeptide/ribosome/mRNA complexes are formed, it may be beneficial to stabilise these complexes using standard translational inhibitors such as chloramphenicol. Additional chemical or photochemical cross-linking of the polypeptide chain and/or mRNA to the ribosome may also be beneficial. For example, the enzyme transglutaminase may be employed using a C-terminal glutamine placed on the nascent polypeptide chain to provide a substrate for cross-linking to a free amino group preferably provided at the 3' end of the RNA molecule using a complementary synthetic oligonucleotide. As an alternative example, chemically-modified amino acids (such as selenium amino acids) might be incorporated into the protein chain for subsequent reaction with chemically modified RNA nucleotides, such as thiolated nucleotides.

Once polypeptide/ribosome/mRNA complexes are formed, the mixture is then screened for binding to the target ligand in such a manner that successfully binding complexes can subsequently be recovered. This could be achieved by virtue of binding to a target ligand immobilised onto a solid phase such as a plastic or glass surface or the surface of a latex or magnetic bead. This could also be achieved by virtue of the binding of the target ligand effecting another reaction or series of reactions which subsequently provides the basis for separation of the complex, for example where binding to the target ligand on the surface of a cell causes a change in that cell such as the appearance of a surface antigen whereby the cell can be separated from other cells in the population along with the bound polypeptide/ribosome/mRNA complex. As an alternative, the polypeptide could be screened for a particular enzymic activity whereby the target ligand is, for example, an enzyme substrate which irreversibly binds to the enzyme or where the substrate is converted by the enzyme to a product which can combine with the polypeptide/ribosome/mRNA complex or can effect some other change such that the polypeptide/ribosome/mRNA complex including the enzymic activity can be separated from the total mixture of complexes.

In order to avoid non-specific binding to the target ligand, various blocking agents would be added to the polypeptide/ribosome/mRNA complexes for incubation with the target ligand, especially to avoid non-specific associations of the mRNA with the target. Whilst various standard blocking agents such as serum albumin or casein might be beneficial, a preferred method which specifically blocks non-specific mRNA binding is to form the cDNA prior to binding the polypeptide/ribosome/mRNA complexes using a synthetic oligonucleotide primer and reverse transcriptase. The use of this step also provides protection for the mRNA from enzymic degradation whilst, after binding to the target ligand, permitting subsequent enzymatic destruction of the mRNA using RNaseH which degrades mRNA in a mRNA:cDNA hybrid.

Once polypeptide/ribosome/mRNA complexes are separated by virtue of binding to a target ligand, the complexes can either be purified preparatively by standard methods or the RNA simply released using, for example, EDTA or simple heating prior to reverse transcription into cDNA and, preferably, amplification by methods such as PCR. Alternatively, the mRNA could be copied into cDNA prior to binding of the polypeptide/ribosome/mRNA complexes and then destroyed with RNaseH as above. The amplified DNA can then either be transcribed and translated again in order to further divide the library or can be cloned to produce a sublibrary. For molecular evolution strategies where polypeptides which bind to the target ligand are evolved by mutation, mutagenesis can either be effected at the stage of cDNA amplification using, for example, error-prone PCR or can be effected by subjecting the cloned sublibrary to mutagenesis, for example using mixed oligonucleotides to mutagenise specific regions of the polypeptide-encoding DNA segment. If required, the initial DNA library for transcription/translation or a sublibrary following screening can be subdivided into pools or individual clones for screening in order to reduce the complexity of the mixture of polypeptides subjected to screening. After finally identifying one or more polypeptides deriving from the DNA library, DNA encoding the specific polypeptides of interest can then be subcloned into expression vectors in order to produce high levels of the desired protein without, as required, the spacer segment or any other flanking segments.

It will be apparent to those skilled in the art that the present invention will have a variety of applications including the isolation of variants of known polypeptide types such as single-chain antibodies or totally novel proteins with biochemical or biological activities superior to those of existing proteins. Chimaeric proteins created as hybrids of regions from known polypeptides and regions of novel polypeptide sequence may also be produced. For multichain polypeptide types such as antibodies, functional or novel combinations of heavy and light chain variable regions may also be produced.

In another embodiment of the present invention, protein-ribosome-mRNA complexes are produced whereby the protein portion of the complex is embedded in a membrane in order to contribute to the proper structure of the protein, especially where the protein is a membrane-bound receptor. This can be achieved by methods such as by producing artificial membranes with the protein-ribosome-mRNA complex present such as by producing liposomes whereby the liposome is formed with the protein embedded in the membrane. On other cases, the protein might be produced either within or outside a vesicle such as a liposome such that the protein subsequently becomes embedded in the membrane. By means such as this, receptors may be

displayed on the surface of vesicles or artificial membranes or even manipulated cells (such as red cell 'ghosts') whereby such receptors can be used as a means for selecting ligands which bind to these receptors or can be used in order to determine interactions of the receptor with other proteins such as intracellular 'second messenger' proteins as part of a signalling pathway from the receptor. In cases where a mixture of protein-ribosome-mRNA complexes from a cDNA library are produced with the protein receptor portion of the complex is embedded in a membrane, then selection of such receptors may be achieved by using the ligand to isolate vesicles or membranes displaying the receptor thus, by virtue of the attached mRNA, providing for a rapid means of identifying the DNA sequence encoding the receptor.

The following examples are provided to illustrate the invention and should not be considered as limiting the scope of the invention.

Example 1

The starting point was two single-chain antibody genes (scFv) specific for the epidermal growth factor receptor (EGFR) and for the prostate surface membrane antigen (PSMA) each cloned into an E.coli expression vector, pPMhis. The scFv fragments were recloned into a pGEM T7/SP6 plasmid (Promega, Southampton, UK) providing an upstream promotor for T7 RNA polymerase using long synthetic oligonucleotides and PCR to provide an upstream bacterial ribosome binding site, a downstream spacer segment derived from the M13 phage gene III and a 3' transcriptional termination region from the E.coli lpp terminator. For both scFv's, the translational stop codon was removed by the PCR reaction. The resultant plasmids were called pT7340A (for EGFR) and pT7591A (for PSMA).

As variants of the basic plasmids above, an upstream anti-initiation segment and a downstream terminating segment were provided by SOE PCR to insert, respectively, a secretory leader sequence between the ATG initiation codon and the scFv genes, and a terminal sequence TAR sequence between the spacer segment and the transcriptional termination sequence. The resultant plasmids were called pT7340B (for EGFR) and pT7591B (for PSMA).

In vitro transcription of the pT7 plasmids was performed using a RiboMAX™ kit (Promega, Southampton, UK) according to the manufacturer's instructions. The resultant mRNA was purified according to the manufacturer's protocol.

In vitro translation was performed in an E.coli S-30 system as described by Chen and Zubay (ibid) modified as described by Hanes and Pluckthun (ibid) and supplemented with HIV Tat 37-72 peptide. After 10 minutes of translation, a preparation of SRP (produced by the method of Romisch K, Webb J, Herz J, Prehn S, Frank R, Brenner S and Walter P, Nature vol 340 (1989) p478-482) was added and the translation was continued for a further 10 minutes. The translation was stopped and the mixture centrifuged as described by Hanes and Pluckthun (ibid). The translation reactions were then incubated for 1 hour at room temperature on microtitre plates with either EGFR or PSMA coated into the plates. Washing and dissociation of retained ribosome complexes, isolation of mRNA, reverse transcription-PCR and repeated transcription-translation were as described by Hanes and Pluckthun (ibid). After 5 rounds of

ribosome display, the PCR products were cloned into pUC18 for sequence determination and determination of the representation of the original scFv's in the selected population of genes. For this determination, the inserts in at least 50 pUC18 clones were sequenced.

For screening on an EGFR antigen preparation, an original plasmid mixture comprising a 1:1 molar ratio of pT7340A : pT7591A gave rise to a final pUC18 library consisting of 84% pT7340A sequence and 16% pT7591A. With a 1:1 molar ratio of pT7340A : pT7340B or a 1:1 molar ratio of pT7340B : pT7591B, the final pUC18 library comprised 100% of the pT7340B sequence in both cases.

Example 2

The starting point was two single chain antibody (scFv) genes, one specific for digoxin (Tang *et al.*, Journal of Biological Chemistry 270 (1995) p7829-7835) and the other for fluorescein (Denzin & Voss, Journal of Biological Chemistry 267 (1992) p8925-8931). The anti-digoxin scFv was PCR amplified from the pCANTAB vector (Tang *et al.*) using forward primer, fdig1: CCG TAT AGA TCT CAG GTC AAA CTG CAG GAG TCT and reverse primer, rdig1: CCG TAT GGA TCC CCG TTT TAT TTC CAA CTT TGT. PCR amplification reactions were performed using Boehringer Expand High Fidelity PCR system. Reaction conditions for amplification of DNA fragments were 1X Expand HF buffer, 2.5 mM MgCl₂, 4 mM of each dNTP, 2.5 units of polymerase, 10 ng template DNA and 30 pmol of primer DNA. Reactions were incubated in a thermal cycler using the following programme: 92°C for 5 min, 53-67°C (depending on primer sequence) for 5 min, 72 °C for 1 min, followed by 30 cycles of 92°C for 1 min, 53-67°C for 1 min and 72°C for 1 min. The resultant 720 bp fragment was then purified using Wizard PCR purification columns (Promega) and cloned into the *E. coli* expression vector pET-9 (Promega) at the BamHI site by digesting the PCR product and vector with *Bgl*II and *Bam*HI according to the manufacturers instructions. This plasmid contains the promoter, translational start site and terminator from the bacteriophage T7 gene 10. The resultant plasmid was designated pDIG1. The anti-fluorescein scFv DNA sequence was generated as described by Mallender, Carrero & Voss (Journal of Biological Chemistry 271 (1996), p5338-5346) from the expression vector pGX8773 using forward primer, fox1: CCG TAT AGA GAT GTC GTG ATG ACC CAA ACT and reverse primer, rox1: CCG TAT GGA TCC TGA GGA GAC GGT GAC TGA GGT. This fragment was generated by PCR and then cloned into the pET-9 vector as described above and designated pOX1.

A spacer sequence based on the glycine rich linkers of gene III of filamentous phage M13 was generated by performing PCR on a preparation of double stranded M13 DNA using the following two sets of primers:

m13f1: CCG TAT AGA TCT GGCTTAATGAGGATCCATT
 *Bgl*II

m13r1: CCG TAT CTC GAG CTGTAGCGCGTTTCATCGGC
 *Xho*I

m13f2: CCG TAT GTC GAC GGCTTAATGAGGATCCATT

*Sal*I

m13r2: CCG TAT TGA TCA CTGTAGCGCGTTTCATCGGC
*Bcl*II

Two sets of PCR reactions were performed using primer combination m13f1 and m13r1 or with m13f2 and m13r2. These two sets of reactions generated two populations of products, one with a 5' *Bg*II and 3' *Xho*I and one with a 5' *Sal*I and 3' *Bcl*II restriction sites. The restriction sites were included to facilitate the construction of multimers of the 30 amino acid linker. The *Bg*II / *Xho*I PCR products were double digested and phosphatased and then ligated with the digested *Sal*I/*Bcl*II PCR products. In this way multimers ligated only 5' to 3' (which could be confirmed by digestion) would be formed. A 900 bp fragment was isolated by agarose gel electrophoresis and purified using Wizard PCR purification columns (Promega) according to manufacturers instructions. The fragment was then digested with *Bg*II and *Bcl*II and cloned into the *Bam*HI site of pDIG1 and pOX1 downstream of the scFv fragments to generate pDIG2 and pOX2 respectively.

As variants of the basic plasmids above, an upstream anti-initiation segment was inserted between the AUG initiation codon and the scFv genes. This was performed by incorporating a murine Vh signal sequence (Neuberger, EMBO J. 2 (1983) p1372-1378) to the scFv PCR primers fdig1 and fox1:

fdig2: CCG TAT AGA TCT ATG GGA TGG AGC TGT ATC ATC CTC TTC TTG
GTA GCA ACA GCT ACA GGT GTC CAC TCC CAG GTC AAA CTG CAG GAG
TCT

fox2: CCG TAT AGA TCT ATG GGA TGG AGC TGT ATC ATC CTC TTC TTG
GTA GCA ACA GCT ACA GGT GTC CAC TCC GAT GTC GTG ATG ACC CAA
ACT

Following PCR in combination with rdig1 and rox1 respectively, the resultant PCR products were digested with *Bg*II and *Bam*HI and cloned into the pET vector as previously described to generate pDIG3 and pOX3.

In addition the HIV transactivation response element (TAR) sequence was inserted downstream from the M13 spacer segment by ligating the *Bcl*II digested 900 bp spacer fragment (generated as described above) to a self annealed oligonucleotides encoding the HIV TAR as follows:

TAR1: GATCAGCCAGATTGAGCAGC
TAR2: GATCGCTGCTCAAATCTGGCT

The fragment was repurified and cloned into the *Bam*HI site of pDIG3 and pOX3 to generate pDIG4 and pOX4.

Following cloning, sequencing of the PCR generated inserts was performed by the dideoxy chain termination method using a double-stranded plasmid DNA template (Kraft *et al.*, BioTechniques 6 (1988), p544) and Sequenase (Amersham) using T7

sequencing primers.

In vitro transcription of the constructs pDIG3, pOX3 and pDIG4, pOX4 was performed using the RiboMAX large scale RNA production system (Promega) according to the manufacturers instructions. The resultant mRNA was purified using PolyATtract system (Promega).

In vitro translation with mixtures of anti-digoxin and anti-fluorescein scFv mRNAs were performed in an *E. coli* S-30 system as described by Chen and Zubay (*ibid*) modified as described by Hanes and Pluckthun (*ibid*) and in the presence or absence of 10ug/ml HIV tat 37-72 peptide (Naryshkin *et al.*, Biochemistry 36 (1997), p3496-3505). In some samples, a preparation of SRP (produced by the method of Romisch *et al.*, Nature 340, (1989), p478-482) was added after 10 minutes of translation and the translation continued for a further 10 minutes. The translation was stopped and the mixture centrifuged as described by Hanes and Pluckthun (*ibid*). The translation reactions were then incubated for 1 hour at room temperature on microtitre plates with either digoxin or fluorescein coated onto the plates. Washing and dissociation of retained ribosome complexes, isolation of mRNA, reverse-transcription PCR and repeated transcription-translation were as described by Hanes and Pluckthun (*ibid*). After 5 rounds of ribosome display, the PCR products were cloned into pUC18 for sequencing and determination of the original scFv's in the selected population of genes. For this determination, the inserts in at least 50 pUC18 clones were sequenced.

The results of this analysis are shown in table 1 which indicates that translation reactions with added tat resulted in an increased selection of mRNAs encoding scFv's against the target ligand with some indication of increased selection with added SRP.

Example 3

The human IL-5 protein was used to demonstrate that ribosome display can be used to identify interacting proteins by the use of dicistronic constructs. Three basic expression plasmids were constructed as detailed below;

Construct 1

The human IL-5 gene was PCR amplified from pUC 18 (as supplied by R&D Systems, Abingdon, UK) using the following primers:

il5f1: CCG TAT AGA TCT GAA ATT CCC ACT AGT GCA TTG
BglII

il5r1: CCG TAT GGA TCC GAC GTC CTC AAG CTT GGA ATA TTA TCA
BamHI *HindIII* stop

GTG ATG GTG ATG GTG ATG ACT TTC TAT TAT CCA
His tag

PCR amplification reactions were performed using Boehringer Expand High Fidelity PCR system (Boehringer, Lewes, UK). Reaction conditions for amplification of DNA fragments were 1X Expand HF buffer, 2.5 mM MgCl₂, 4 mM of each dNTP, 2.5 units of polymerase, 10 ng template DNA and 30 pmol of primer DNA. Reactions were incubated in a thermal cycler using the following programme: 92°C for 5 min, 53-67°C (depending on primer sequence) for 5 min, 72 °C for 1 min, followed by 30 cycles of 92°C for 1 min, 53-67°C for 1 min and 72°C for 1 min. The resultant 448 bp fragment was then purified using Wizard PCR purification columns (Promega) and cloned into the *E. coli* expression vector pET-9 (Promega) at the *BamHI* site by digesting the PCR product and vector with *BglII* and *BamHI* according to the manufacturers instructions. This plasmid contains the promoter, translational start site and terminator from the bacteriophage T7 gene 10. The resultant plasmid was designated pIL5a.

Construct 2

The human IL-5 gene was PCR amplified from pUC18 using the following primers:

il5f2: CCG TAT AGA TCT AAG CTT GAA ATT CCC ACT AGT GCA TTG
BglII *HindIII*

il5r2: CCG TAT GGA TCC ACT TTC TAT TAT CCA CTC GGT
BamHI

PCR amplification and purification were performed as described above. The PCR product was then digested with *BglII* and *BamHI*, cloned into the *BamHI* site of the pET9 vector (Promega) and designated pIL5b.

A spacer sequence based on the glycine rich linkers of gene III of filamentous phage M13 was generated by performing PCR on a preparation of double stranded M13 DNA using the following two sets of primers:

m13f1: CCG TAT AGA TCT GGCTTAATGAGGATCCATT
BglII

m13r1: CCG TAT CTC GAG CTGTAGCGCGTTTCATCGGC
XhoI

m13f2: CCG TAT GTC GAC GGCTTAATGAGGATCCATT
SalI

m13r2: CCG TAT TGA TCA CTGTAGCGCGTTTCATCGGC
BclI

Two sets of PCR reactions were performed using primer combination m13f1 and m13r1 or with m13f2 and m13r2. These two sets of reactions generated two populations of products, one with a 5' *BglII* and 3' *XhoI* and one with a 5' *SalI* and 3' *BclI* restriction sites. The restriction sites were included to facilitate the construction of multimers of the 30 amino acid linker. The *BglII* / *XhoI* PCR products were double digested and phosphatased and then ligated with the digested *SalI/BclI* PCR products. In this way multimers ligated only 5' to 3' (which could be confirmed by digestion) would be formed. A 900 bp fragment was isolated by agarose gel electrophoresis and purified using Wizard PCR purification columns (Promega) according to manufacturers instructions.

The HIV transactivation response element (TAR) sequence was inserted downstream from the M13 spacer segment by ligating the *BclI* digested 900 bp spacer fragment (generated as described above) to a self annealed oligonucleotides encoding the HIV TAR as follows:

TAR1: GATCAGCCAGATTGAGCAGC
TAR2: GATCGCTGCTCAAATCTGGCT

The fragment was repurified, digested with *BglII* and cloned into the *BamHI* site of pIL5b to generate pIL5c.

Construct 3

pIL5c was digested with *HindIII* and *BamHI* to release the insert described in construct 2 and this was then cloned in to pIL5a which also had been digested with *HindIII* and *BamHI*. The resultant construct was designated pIL5d.

Following cloning, sequencing of the PCR generated inserts was performed by the dideoxy chain termination method using a double-stranded plasmid DNA template (Kraft *et al.*, BioTechniques 6 (1988), p544) and Sequenase (Amersham, Little Chalfont, UK) using T7 sequencing primers.

In vitro transcription of the constructs pIL5a, pIL5c and pIL5d was performed using the RiboMAX large scale RNA production system(Promega) according to the manufacturers instructions. The resultant mRNA was purified using PolyATract system (Promega).

In vitro translation was performed in an *E. coli* S-30 system as described by Chen and Zubay (*ibid*) modified as described by Hanes and Pluckthun (*ibid*) and supplemented with HIV tat 37-72 peptide (Naryshkin *et al.*, *Biochemistry*, vol 36 (1997), p3496-3505). The translation was stopped and the mixture centrifuged as described by Hanes and Pluckthun (*ibid*). Purification of the translation mix was performed under denaturing conditions using the rapid affinity column chromatography with the pET-His-Tag system as described by Novagen (Cambridge Biosciences, Cambridge, UK). Bound proteins were eluted according to manufacturers instructions.

Anti-tat antibody was made using the tat peptide 37-72 which was conjugated via its N-terminal cysteine residue to KLH using MBS according to *Antibodies, A Laboratory Manual* *ibid*. 10ug of the conjugate was used to immunise Balb/c mice as above and serum was collected and used at 1:100 dilution in the further experiments. Dilutions of pET-His-TagA-eluted material from above was applied to an Immulon 2 96-well microtitre plates (Dynatech, Chantilly, VA, USA) according to the manufacturer's instructions and anti-tat antibody was then added and incubated for 2 hours at room temperature. Plates were then washed 3 times with PBS, and a 1:1000 dilution of HRP-labelled goat anti-mouse antibody conjugate (#A4416, Sigma, Poole, UK) was added and incubated for a further 1 hour at room temperature. After 3 washes in PBS, TMB substrate was added according to *Antibodies, A Laboratory Manual* *ibid*.

The results showed that the eluted His-tagged protein was associated with tat protein in ELISA assays derived from the dicistronic plasmid pIL5d but not from a 1:1 mixture of monocistronic plasmids pIL5a and pIL5c where no bound tat was detected. This indicated that translated IL-5 had homodimerised from the dicistronic plasmid but not from the monocistronic plasmids.

Example 4

The starting point was a human scFv library (Nissim *et al.*, *EMBO J.* 13 (1994), 692) and the vector RD1. The RD1 vector was based on a pET-5 vector (Promega, Southampton, UK) and was created by using site directed mutagenesis to insert *Nco*I and *Not* I restriction sites downstream of the ATG initiation codon. The RD1 vector provides a ribosome binding site and a T7 promoter and terminator. A downstream spacer segment derived from the M13 phage gene III was generated as described in example 2 and cloned into the RD1 vector at the *Bam*H I site to create the RD1a plasmid.

The scFv genes were cloned into the RD1a vector as follows. The scFv inserts were PCR amplified using the following primers which also incorporate a murine Vh signal sequence:

fsc1: CCG TAT GAT CCA TGG GGA TGG AGC TGT ATC ATC CTC TTC
TTG GTA GCA ACA GCT ACA GGT GTC CAC TCC TCG CGG CCC AGC CGG
GCA TGG .

rsc1: GAG TTT TTG TTC TGC GGC CGC

The resultant PCR products were digested with *Nco*I and *Not*I and cloned into the RD1 vector which was also digested with *Nco*I and *Not*I.

The HIV transactivation response element (TAR) was inserted downstream from the M13 spacer segment as described in example 2.

In vitro transcription was performed using a RiboMAXTM kit (Promega, Southampton, UK), the transcripts capped and purified according to manufacturer's instructions.

In vitro translation was performed using the Rabbit Reticulocyte Lysate system (Promega, Southampton, UK) according to the manufacturers instructions with the addition of HIV Tat 37-72 peptide to the translation mixture. The translation reaction was incubated at 30°C for 50 minutes. A preparation of SRP (see example 1) was added and the translation reaction continued for a further 10 minutes.

Magnetic beads (Dynal) were coupled to NIP (4 hydroxy-5-iodo-3-nitrophenylacetyl) and fluorescein and added to the translation mixture. The reaction was incubated at 4°C for 60 minutes with gentle vibration. The beads were recovered and heated to 80°C for 5 minutes and then plunged onto ice to dissociate the scFv-ribosome-mRNA complex. A 2 ul aliquot of the bead suspension was then used to generate cDNA as described by Hanes and Pluckthun. After 5 rounds of ribosome display the cDNA's were cloned into pUC18. 20 clones were selected for further analysis and sub-cloned into the pPM1-His vector. The scFv's were then expressed as SCAB's as described by McGregor *et al.* and their binding affinities determined by ELISA.

Of the 20 clones 12 showed binding to fluorescein and 8 showed binding to NIP.

Ligand

dig/fluor mRNA ratio	tat	SRP	Digoxin % of anti-dig scFv clones	Fluorescein % of anti-FITC scFv clones
1:99	-	-	18	n/a
	+	+	96	n/a
5:95	-	-	26	n/a
	+	-	94	100
	+	+	88	100
50:50	-	-	82	n/a
	+	-	100	100
	+	+	100	n/a
95:5	-	-	n/a	36
	+	-	100	94
	+	+	n/a	98
99:1	-	-	n/a	28
	+	-	100	100
	-	+	n/a	40
	+	+	n/a	94

n/a = not measured

Table 1:

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